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R le of interacti ns at the lipid-wat r interface for domain formation

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Summary

The lipid-water interface is critical for the packing of lipid molecules in membranes. We have demonstrated that lateral phase separation in membranes can be driven by electrostatic interactions such as those involving charged lipid species and ppositely charged peptides, in addition to hydration effects at the lipid-water interface. By using nuclear magnetic resonance (NMR), circular dichroism and fluorescence spectroscopy we have shown that binding of a 21-amino acid peptide containing six positively charged arginine residues to mixed phosphatidylcholine (PC)/phosphatidylglycerol (PG) membranes results in a conformational change in the peptide from a random coil to a helical structure and causes the formation of domains of negatively charged PG. Binding of the peptide to PG membranes disorders the lipid hydrocarbon chains. The strength of lipid-peptide binding at the interface, the conformational change in the peptide, and domain formation with the negatively charged lipid are coupled energetically. The lipid-peptide association constant is lower for membranes containing 20 mol% PG in PC/PG mixtures than for 100% PG membranes. We suggest that one of the factors that lower the association constant in PC/PG membranes is entropic energy of formation of PG domains. Besides electrostatic interactions, hydration of lipids is important for domain formation. We have shown that dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylethanolamine separate under conditions of decreased water activity. Furthermore, water activity controls lipid packing stress in the hydrocarbon core and the headgroups of membranes as demonstrated by induction of an inverse-hexagonal-to-lamellar phase transition in dioleoylphosphatidylethanolamine. The experiments have shown that lipid-peptide and lipid-water interactions at the interface influence the packing of lipid hydrocarbon chains. Consequently we predict that a change in lipid-lipid interaction in the hydrocarbon core of the membrane, for example as a result of the introduction of polyunsaturated fatty acids, will alter lipid-solvent and lipid-peptide interactions at the interface.

Keywords: Domain, phospholipid, lipid-peptide interaction, hydration, hexagonal phase.

Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DMPC; 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPC-d_{54}, chain perdeuterated DMPC; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt); DMPG-d_{54}, chain perdeuterated DMPG; DOPG, 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt); DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; P828, peptide with the amino acid sequence RVIEVVQGACRAIRHIPRRIR; PEG 20 000, polyethylene glycol (M_r = 20 000).

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Introduction

There are numerous indications that the lipid environm nt is critical for the function of membrane proteins (e.g. Selinsky 1992 and referer.ces therein). The lipid composition of the annulus around a protein which, operationally, we may call a lipid domain, deviates from the composition of the bulk lipid phase. At equilibrium, domain formation is driven by enthalpic contributions to the free energy. However, for entropic reasons the free energy of a non-randomly mixed lipid syst m is higher than it would be for a random distribution. Th energy of binding of the protein to the bilayer, the energy of lipid domain formation upon binding, and the energy of structural transitions of the protein at or in the membrane are contributing to the free energy. As a result, these events are energetically coupled. In this context domain formation may influence the strength of protein binding, and lipid composition may influence protein activity.

We investigated lipid-protein interaction by studying the interaction of a model peptide derived from the carboxy-terminus of the envelope glycoprotein gp160 of HIV-1, amino acid sequence RVIEVVQGACRAIRHIPRRRIR (abbreviated P828), with mixed phosphatidylcholine (PC)/phosphatidyl-glycerol (PG) bilayers. Peptide binding was analysed in terms of an association constant using the ζ-potential from micro-electrophoresis measurements as a quantitative measure for the amount of bound peptide. Using fluorescenc spectroscopy with covalently labelled lipid and nuclear magnetic resonance (NMR), we investigated changes in lipid mixing and membrane structure. Results on the structural transition of P828 upon binding are presented in the paper of Koenig *et al.* 1994, in this issue.

One mechanism for the control of lipid-lipid and lipid-protein interactions involves interactions at the lipid-water interface. Operationally we define this region as the locations where water interacts with lipids, which includes the entire lipid headgroup, the glycerol backbone, the carbonyl- and uppermost methylene groups of lipid acyl chains. Order parameters in phospholipid membranes are highest for the glycerol backbone/upper hydrocarbon chain region and decrease rapidly towards the lipid headgroups and the terminal methyl groups of the chains (Seelig 1977, Bloom *et al.* 1991). Phospholipid membranes are most tightly packed where the molecular order parameters are high. The area per lipid molecule in a monolayer at the level slightly below the glycerol remains almost unchanged even when the lipid water dispersion converts into non-lamellar phases (Rand *et al.* 1990, Chung and Caffrey 1994). The question arises: Why do lipids pack so tightly at the glycerol backbone? Presumably packing of lipids in this region has to be precise in ord r to seal the lipid bilayer against exc ssive hydrocarbon-water contact which is energetically unfavourable (Tanford 1980).

The tight packing of lipid molecules at the glycerol forc s the hydrocarbon chains, and to some xtent the lipid

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headgroups, to align. This alignment is entropically unfavourable. Apparently the structure of the interface and the hydrocarbon interior f membranes are linked. We have demonstrated that interactions at the interface have an influence on the packing of hydrocarbon chains in the center of the bilayer. If the need for tight packing at the interface is eased, e.g. as a result of the binding of a peptide, then both the chains and the headgroups may disorder. We have studied the influence of peptide binding on lipid order by ^{31}P- and ^{2}H NMR on deuterated lipids.

The key to understanding the lipid-water interface lies in the interaction of water with the membrane surface. Surface water is structurally different from bulk water (Finer and Darke 1974) even though it is in fast exchange. Packing of lipid headgroups is distinctive because the volume they occupy is shared also with water of hydration. The lipid-water interaction explains the sensitivity of structural transitions of lipids to water activity. We investigated lateral phase separation in DPPC/DPPE mixtures as a function of water activity.

An alternative means of achieving a more random orientation of lipid chains and thus relaxing entropic constraints of chain packing would be to adopt a phase state, with highly curved lipid monolayers, such as the inverse hexagonal (H_{II}) phase. The area per molecule at the level of headgroups and chains is rather different in lamellar and H_{II} phases. An increase of the area per end of the hydrocarbon chain, while keeping the area constant close to the glycerol, r sults in a decrease of the area per headgroup and consequently in the space available for water for hydration. The influence of water activity on transitions between lamellar and H_{II} phases was investigated on DOPE water dispersions.

Results

Interaction of a peptide from the carboxy terminus of gp160 with neutral and charged phospholipids

Interaction of the peptide P828 with the negatively charged PG at a lipid to peptide ratio of 12/1 resulted in a 20% decrease of the chemical shift anisotropy of the lipid phosphate signal, and a substantial decrease in order parameters of the lipid chain methylene groups (Figure 1). Addition of 20 μM P828 to DOPC/DOPG membranes containing anthrylvinyl-labelled PG resulted in an increase of fluor scence polarization from 0·084 to 0·126. Peptide binding to DMPG bilayers had a profound influence on the gel-to-liquid-crystalline phase transition of the lipid. While the width of the phase transition for the pure lipid is less than 1°C, addition of peptide at a molar ratio of 12/1 results in a transition width of more than 20°C. The temperature of the midpoint of the transition with and without peptide was approximately identical.

The peptide P828, at concentrations of up to one peptide molecule per 10 lipid molecules, did not change either the chemical shift anisotropy of the lipid phosphate signal, the quadrupolar splittings of perdeuterated acyl chains, or the gel-to-liquid-crystalline phase transition temperature of zwitterionic PCs. This is in agre ment with fluorescence polarization measurements on anthrylvinyl-labelled PC which did not indicate any interaction with the peptide.

Domain or cluster formation in mixed DOPC/DOPG bilayers

Domain formation was investigated using anthrylvinyl-labelled PG and perylenoyl-labelled PC (Figure 2). Addition of the peptide resulted in a decrease of fluorescence energy transfer from anthrylvinyl to perylenoyl. Furthermore, if the anthrylvinyl-labelled PG was present at an initial concentration of 1 mol%, the addition of 20 μM P828 resulted in self-quenching of anthrylvinyl. Both observations are consistent with the formation of areas in the bilayer with significantly increased PG concentration. We did not interpret the data in terms of domain or cluster sizes and composition because of the strong model dependence of a theoretical analysis.

Determination of lipid-peptide association constants

Lipid-peptide association constants were determined by measuring the microelectrophoretic mobility of multilamellar liposomes. For simplicity it was assumed that the ζ-potential

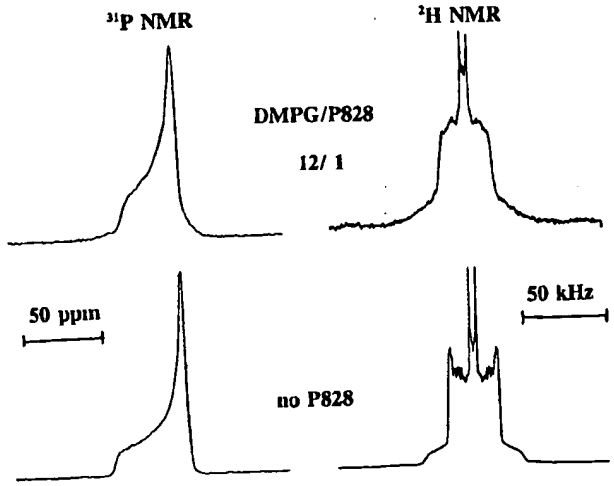

Figure 1. ^{31}P and ^{2}H NMR spectra of DMPG-d_{54} at 22°C with and without addition of peptide P828 (molar ratio lipid/peptide = 12/1). Addition of the peptide reduces the anisotropy of chemical shift of lipid phosphate groups and lowers the order parameters of fatty acid methylene groups.

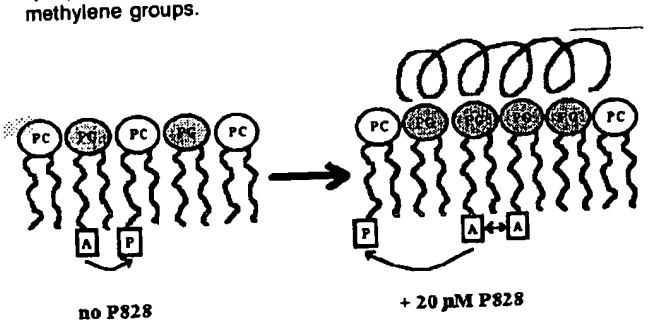

Figure 2. Addition of a 20 μM peptide P828 resulted in the formation of domains or clusters enriched in PG as observed by concentration-self-quenching of anthrylvinyl-labelled PG and a reduced fluorescence energy transfer from anthrylvinyl-PG to perylenoyl-PC (see Gawrisch *et al.* 1993 for experimental details).

is identical to th electrical surface potential. Th measurements were perform d as a function of the concentration of negatively charged DOPG in DOPC/DOPG mixtures and of P828 concentration. The negatively charged membrane surface attracts the positively charged P828. The surface conc ntration of P828 depends exponentially on the amplitude of the electrical surface potential. Some of the peptide molecules in the surface layer bind to lipids and neutralize the negative lipid charge. This binding results in a decrease in the microelectrophoretic mobility which can be easily measured. Data were analysed in terms of a model suggested by McLaughlin and co-workers (Kim *et al.* 1991). The lipid-peptide association constant for binding to DOPG membranes in 100 mM NaCl solution is $\simeq 1 \times 10^6$. Association constants for binding to mixed DOPC/DOPG bilayers were significantly decreased. For 20 mol% DOPG in DOPC, a value of $\simeq 2 \times 10^3$ was determined. The association constants are based on peptide concentrations at the surface and, therefore, do not contain effects caused by a variable membrane surface potential. The decrease in the lipid-peptide association constant with decreasing DOPG concentration reflects structural changes in the lipid matrix and the lipid-peptide complexes.

Influence of water activity on lipid mixing

The influence of water activity (a_w) on lipid mixing in binary lipid mixtures was investigated using DPPC/DPPE membranes. The amount of water between lipid bilayers was controlled by adjusting a_w of the surrounding solution with polyethylene glycol (PEG) of $M_r = 20\,000$. Multilamellar liposomes are not permeable to high molecular weight PEGs and can be dehydrated by the osmotic action of PEG without the need of a separating dialysis membrane. Water will xchange between the lipid and the PEG phase until the a_w in both pools is identical.

The phase diagram of DPPC/DPPE mixtures in excess water as a function of the mole fraction of DPPE and temperature has been reported perviously (Arnold *et al.* 1981, Petrov *et al.* 1982). Analogous experiments were carried out in the presence of a 60 wt% PEG 20 000 solution (Figure 3). The water activity in this PEG solution is $a_w = 0{\cdot}85$ (Gawrisch *et al.* 1988). Using NMR and deuterated water we determined that the polyethylene glycol dehydrates the lipids to water concentrations of approximately seven water molecules per lipid (see also Arnold *et al.* 1983). As a result the phase transition temperatures from the gel to the liquid crystalline phase are elevated. The solidus curves of the phase diagrams with and without PEG indicate separation of both lipids in the gel state. After addition of PEG, the lipids separate in the liquid crystalline phase also, as judged from the liquidus curve (cf. phase diagrams of binary lipid mixtures in the review article of Lee 1977).

Energy of dehydration of lamellar and inverse hexagonal phases

We used the osmotic stress t chnique (Parsegian *et al.* 1986) to determine the work necessary to dehydrate lipid-water dispersions. With ^{31}P NMR and X-rays (Gawrisch *et al.* 1992)

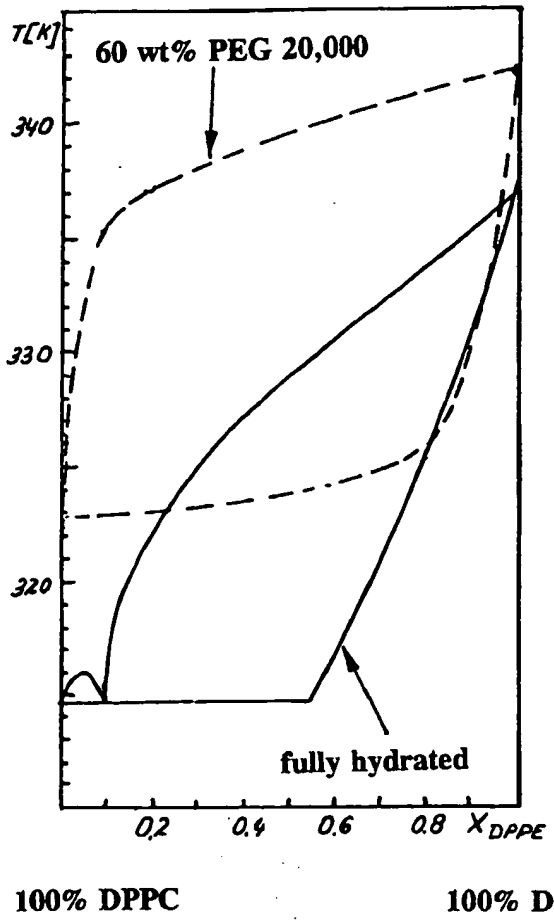

Figure 3. Phase diagram of DPPC/DPPE mixtures as a function of mole fraction of DPPE and temperature as determined by differential scanning calorimetry and NMR. The phase diagram in excess water (solid curves) was published previously (Arnold *et al.* 1981, Petrov *et al.* 1982). The dashed curves are the phase diagram in the presence of 60 wt% PEG 20 000. Dehydration by the PEG solution results in an increase of the gel-to-liquid-crystalline phase transition temperature and in a demixing of both lipids in the gel and liquid crystalline phases.

we determined the phase state of the lipid, and with ^2H NMR the amount of remaining water in both phases. At temperatures above 23°C, the samples remained in the hexagonal phase over the osmotic pressure range from 0 to 20 MPa. Dehydration of the cylindrical micelles of the H_{II} phase results in a continuous change in the radius of curvature of the lipid monolayers. The work to dehydrate the hexagonal phase represents the work necessary to bend the lipid monolayers.

At temperatures below 23°C, dehydration resulted in the formation of a lamellar lipid phase over a limited range of osmotic pressures. At zero osmotic pressure the lamellar lipid phase always contained less water than did the hexagonal phase, but at identical water concentrations it took significantly higher osmotic pressures to dehydrate the lamellar, in comparison to the hexagonal, phase. Integration of osmotic pressure versus water volume curves gives the

work necessary to dehydrate lamellar and hexagonal phases (Figure 4). It takes ~0·5 units of thermal energy, $kT(k = 1 \cdot 38 \cdot 10^{-23}$ J/K, T = 300 K), per lipid molecule (equivalent to 1·2 kJ/mol) to dehydrate the hexagonal phase of DOPE down to about three water molecules per lipid, and on the order of 1·0 kT per DOPE (2·5 kJ/mol) to dehydrate the lamellar phase to the same water volume (Figure 4). However, dehydration to a water volume of three water molecules per lipid is an extreme case. At 22°C, dehydration of the hexagonal phase resulted in a phase transition into a lamellar phase at rather moderate osmotic pressures. The energy per lipid molecule to trigger this phase transition is surprisingly low, just 0·05 kT, or 125 J per mole of lipid.

Discussion

Domain formation triggered by lipid-peptide interaction

Peptide binding was analysed quantitatively in terms of an association constant using microelectrophoresis. With fluor scent-labelled lipid, we detected a non-random distribution of the negatively charged lipid DOPG in DOPG/DOPC mixtures after addition of the peptide. The interaction between peptide and lipid is governed by the binding of the six positively charged arginines to the n gatively charged PG. As shown in the paper by Koenig *et al.* in this issue, the peptide is a random coil in solution and turns into a helical structure upon binding to a negatively charged membrane surface.

Peptide conformation, the strength of peptide binding to the interface, and domain formation in the membrane are energ tically coupled (Figure 5). The energy necessary to turn the peptide into a helix and to form domains of charged lipids in th membrane comes from lipid-peptide interaction. The lipid-peptide association constant is a convenient measure of the changes of free energy in the peptide and in the lipid

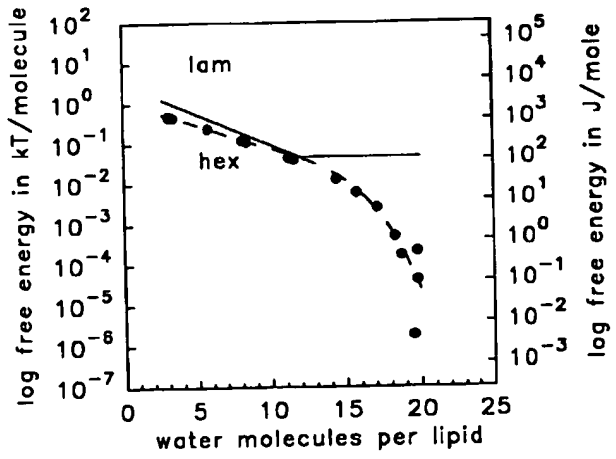

Figure 4. The energy of dehydration of lipid water dispersions increases exponentially with decreasing number of water molecules per lipid. Dehydration of a fully hydrated hexagonal phase of DOPE at 22°C resulted in a transition into a lamellar phase at a water content of around 10 water molecules per lipid. Solid line, lamellar phase (14°C); dashed line, hexagonal phase (22°C).

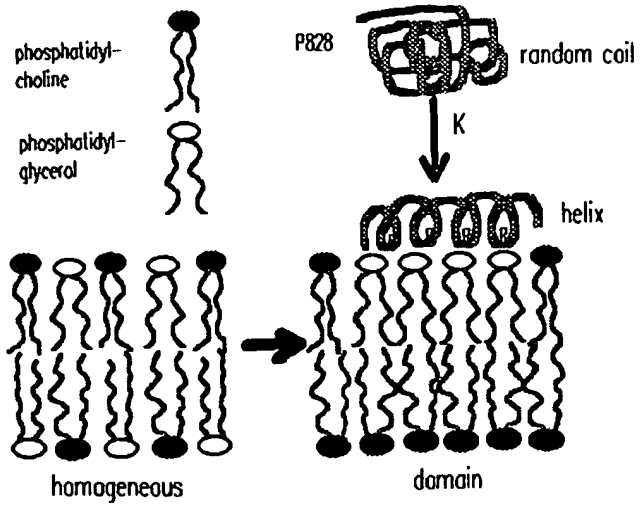

Figure 5. Interaction of the peptide P828 with negatively charged membranes results in a structural transition of the peptide from a random coil to a helical structure (see Koenig *et al.* in this issue). In order to bind to the membrane efficiently the peptide collects the negatively charged PG with its positively charged arginines into domains or clusters. The structural transition of the peptide, in addition to the lipid domain formation, require energy. The lipid-peptide association constant, K, is reflecting the changes of free energies of both peptide and lipids upon binding.

matrix upon binding. Domain formation in membranes is paralleled by a significant decrease in the association constant for binding of the peptide P828. The decrease in the association constant corresponds to a change in the standard free energy of binding of ~16 kJ/mol. If the entire decrease is attributed to changes in the lipid matrix, and if we assume that every arginine of the peptide molecule interacts with a PG molecule, then it takes about 1 kT at room temperature, or 2·5 kJ per mole of PG, to form the domains. This is the correct order of magnitude for the change in mixing entropy for the formation of lipid domains in binary lipid mixtures (Lee *et al.* 1977). Differences in free energies for formation of lipid domains or clusters may result in substantial differences in the amount of bound peptide or protein. Domain or cluster formation depends on lipid acyl chain composition, ionic strength, concentration of divalent cations like Ca^{2+}, water activity, in addition to other factors.

Domain formation triggered by dehydration

In DPPC/DPPE mixtures, dehydration triggered at least a partial separation of lipids in the liquid crystalline phase. The osmotic work of dehydrating the lipid with a 60 wt% PEG 20 000 solution to a water concentration of about seven water molecules per lipid corresponds to 0·1–0·5 kT per molecule or 0·25–1·25 kJ/mol. PC or PE headgroups are surrounded by an inner hydration layer of about five water molecules per lipid. Water molecules in this inner hydration shell contribute an even larger energy to the stability of lipid bilayers. Removal of a water molecule from this shell requires work on the ord r of 1 kT per water molecule or 2·5 kJ/mol of water. Lipid dehydration changes energies of int raction between like and unlike lipids. Separation of lipid species is observed if the

increase in free energy as a result of non-randomness in lipid distribution is ov rcomp nsated by a d crease in energy caused by better matching of lipid–lipid interactions. PCs and PEs have rather different hydration properties (Rand and Parsegian 1989) which may explain th observed separation of lipid species.

Non-lamellar lipid phases

Mod rate dehydration of PE may trigger phase transitions b tween lamellar and non-lamellar phases. The importance of non-lamellar phases for membrane processes is still a matter of discussion. There is no evidence to date for the existence of stable non-lamellar phases in biological membranes. However, the tendency of lipids to form non-lamellar phases has been related to packing stress which may influence the activity of membrane proteins (Seddon 1990 and references therein).

The question arises: How much energy per lipid is n c ssary to trigger transitions between lamellar and non-lamellar phases? The phase transition of DOPE from a fully hydrated inverse hexagonal phase to a lamellar phase at 22°C required just 0·05 kT per molecule (125 J/mol). Phase transitions offer insight into lipid–lipid interactions because they allow us to study perturbations which may appropriate only a few Joules per mole. It is not surprising that there are difficulties seeing these minor perturbations when analysing spectroscopical data on conformation and motions of individual phospholipid molecules. Compared with thermal energies these perturbations are small and do not necessarily change the conformational space associated with the molecule. However, small perturbations per molecule may accumulate in lipid domains. Their significance increases linearly with the number of lipids per domain. Presumably these are the small changes in the membrane environment which are sensed by membrane proteins.

C nclusions

The results confirm the hypothesis that interactions at the lipid–water interface are important for domain formation and for lipid order. Domain formation can be triggered by electrostatic interactions as well as by differences in hydration. We combined structural information from spectroscopy with thermodynamic data such as changes of association constants between lipid and peptide measured as a function of lipid composition, changes of free energies per lipid, and shifts in phase transition temperatures between different lipid phases measured as a function of hydration. Structural data on domain formation in membranes are still incomplete and may get enhanced by a thorough thermodynamical analysis. We have evidence for the existence of packing stress in the hydrocarbon core of membranes which was partly released by the binding of a peptide to the lipid–water interface. Packing stress in the lipid matrix can also be influenced by differences in hydration. Due to the coupling of events at the interface and in the membrane interior, th reverse should be correct as well: interactions betwe n lipids and between lipids and proteins in the hydrophobic core of the membrane may result in a modulation of interactions at the interface.

Experimental pro edures

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), chain perdeuterated DMPC (DMPC-d_{54}), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol amine (DOPE), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DMPG), chain perdeuterated DMPG (DMPG-d_{54}), and 1,2-dioleoyl-*sn*-glycero-3-3[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification. 1,2-Dipalmitoyl-*sn*-glycero-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho ethanolamine (DPPE) were purchased from Fluka (Buchs, Switzerland). Anthrylvinyl-labelled PG and perylenoyl-labelled PC were synthesized in the Shemyakin Institute of Bioorganic Chemistry in Moscow, Russia (Molotkovsky *et al.* 1984).

The peptide P828 was synthesized and purified as described previously (Gawrisch *et al.* 1993). Lipid–water dispersions with lipid/peptide ratios of 12/1 were prepared by brief sonication of lipid and peptide in distilled water. The pH of the dispersion was adjusted to 8·0 with NaOH and HCl. The dispersion was first lyophilized, then transferred into a 4 mm o.d. NMR sample tube, and rehydrated with 50 wt% 150 mM NaCl solution in deuterium-depleted water.

^{2}H and ^{31}P NMR measurements were performed on Bruker MSL-300 and Bruker AMX-360 spectrometers using high power probes with a solenoidal sample coil. ^{2}H NMR spectra were observed with a quadrupolar echo sequence using a pulse delay of 30 μs, and ^{31}P NMR spectra were acquired using a Hahn echo with a pulse delay of 100 μs and strong proton noise decoupling.

The differential scanning calorimetric (DSC) measurements were performed on a Perkin Elmer DSC-2. Weighed amounts of the lipid–water dispersion were sealed in aluminium capsules. Details of the fluorescence spectroscopy experiments and the measurements of microelectrophoretic mobility have been published previously (Gawrisch *et al.* 1993).

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